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14. ABSTRACT

We tested the efficacy of methylselenocysteine (MSC) and finasteride in preventing the clonal expansion of early stage, small volume prostate cancer using a tumor xenograft model. When used alone, MSC had little effect on tumor growth, whereas finasteride was only effective for a short duration. However, the combination was more effective than the single treatments. Due to the small sample size, the observed effects were not statistically significant. A repeat of the experiment with larger sample size is needed to corroborate the findings. We also demonstrated a synergy between emodin and finasteride in suppressing androgen signaling in prostate cancer cells. The combination was more effective in inhibiting cell proliferation and inducing cell death. This provides another option for combined androgen blockade in prostate cancer chemoprevention.

15. SUBJECT TERMS
selenium; 5α-reductase inhibitor; prostate cancer; chemoprevention; androgen receptor; FOXO1A

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A. INTRODUCTION

Androgen plays an important role in prostate carcinogenesis. Testosterone is the major androgen in circulation; it is converted to the more potent dihydrotestosterone in the prostate by the enzyme 5 α-reductase. The Prostate Cancer Prevention Trial (PCPT) demonstrated that treatment with finasteride, an inhibitor of 5 α-reductase, reduced prostate cancer incidence by 25%. Selenium, on the other hand, is shown to reduce prostate cancer risk by 50% by the Nutrition Prevention of Cancer (NPC) trial. However, in October 2008, an interim analysis from the Selenium and Vitamin E Cancer Prevention Trial (SELECT) showed there was no significant reduction of prostate cancer risks in patients supplemented with selenomethionine, either alone or in combination with vitamin E. The controversies regarding SELECT's negative finding for selenium center around the formulation and dose of selenium used in that study. In this project, we propose to use methylselenocysteine (MSC) for the *in vivo* study. MSC is a second-generation selenium compound which has been shown to have excellent anticancer activity *in vivo*. During this period, we focused our effect on the *in vivo* study to test the efficacy of MSC and finasteride in preventing prostate cancer. We also examined the in vitro efficacy of the emodin/finasteride combination in growth inhibition in prostate cancer cells. This part was not included in the Statement of Work, but was conducted to provide further proof regarding the potential for combined androgen signaling blockade in prostate cancer prevention.

B. BODY

Tasks 1 and 3. Determine the optimal dose of finasteride to achieve growth inhibition and assess the combinatorial effect of finasteride and selenium on growth of tumor xenografts in nude mice. We made modifications to the Statement of Work by combining Tasks 1 and 3 because we have found in the literature regarding doses of finasteride that were effective in inhibiting the growth of LNCaP xenografts in nude mice. Based on the literature information, we decided to use finasteride at 5 and 50 mg/kg/day, in combination with MSC at 100 µg/day. For xenografting, $4X10^6$ LNCaP cells were suspended in 50 µl Matrigel (Becton Dickinson Labware) and injected subcutaneously to both sides of the dorsal flank. The Matrigel milieu is required for the formation of tumors in immunodeficient mice [1]. Eighteen mice were randomized into 6 groups (Table 1), with 3 mice per group. MSC and finasteride were administered the day after tumor implantation. Finasteride was prepared in a mixture of 10% ethanol/90% olive oil and given to the mice by oral gavage using a ball-tipped feeding needle. MSC was dissolved in phosphate-buffered saline and administered by intraperitoneal injection. Animals were observed daily, and tumor

Table 1. Description of the treatment groups.

Group ID	MSC (ug/day)	Finasteride (mg/kg)
Control	0	0
MSC	100	0
F5	0	5
F50	0	50
MF5	100	5
MF50	100	50

measurements were taken twice weekly. Tumor volumes were calculated by the following formula: length x width x height x 0.5236. Figure 1A shows the tumor growth curves for up to 8 weeks. MSC treatment did not seem to have any impact on tumor growth. Finasteride at both doses slowed down tumor growth initially, but the inhibitory effects diminished over time. The higher dose of finasteride (50 mg/kg/day) even appeared to stimulate tumor growth after 4 weeks. However, in animals receiving the combination treatments (MF5 and MF50), tumors grew at a slower rate than in control mice, or in mice receiving single treatments. Due to the small samples sizes, these results are not statistically significant ($p>0.05$). The animals were sacrificed after 8 weeks, and the tumor weight data correlate very closely with the tumor volume data. In summary, the tumor growth results suggest that MSC alone does not have a significant impact on inhibiting tumor growth. Rather, it could enhance the efficacy of finasteride and prevent the development of drug resistance to finasteride, implying that MSC may function as a drug modulator.

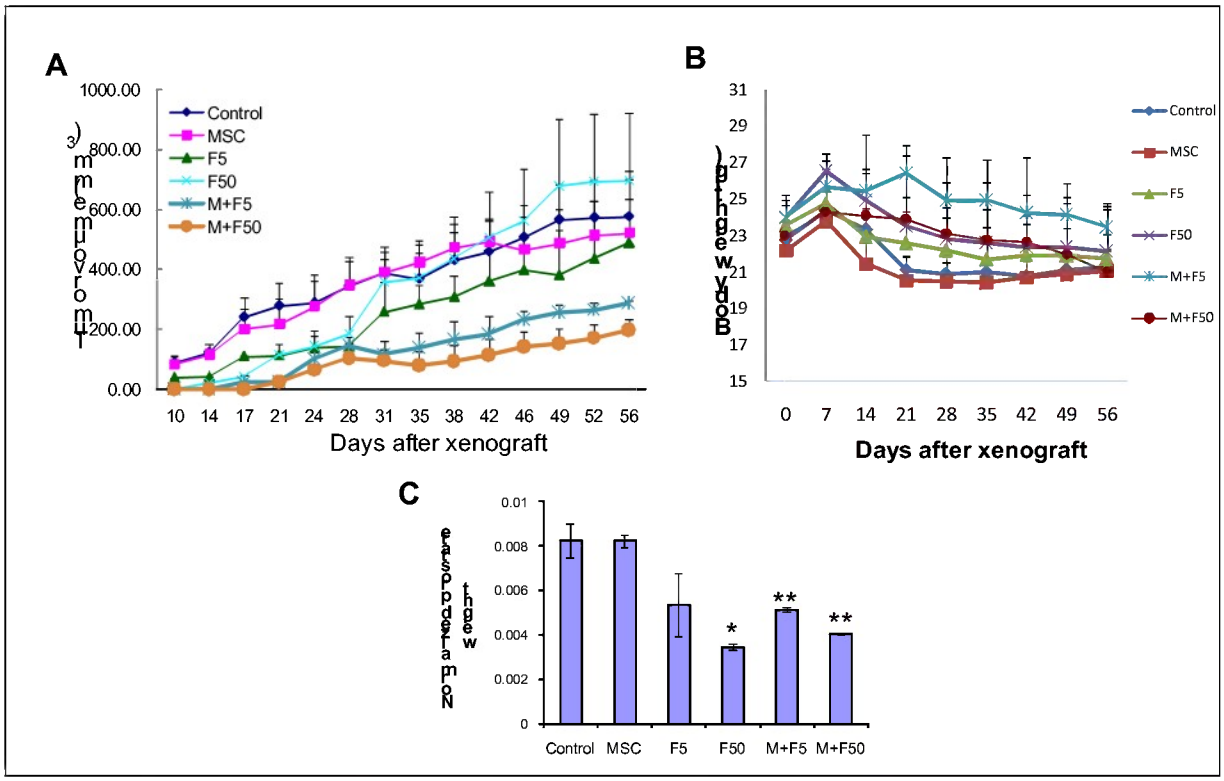

Figure 1. Effect of methylselenocysteine (MSC) and finasteride on LNCaP xenograft in nude mice. A. Tumor growth curve calculated from tumor volume. B. Body weight. C. Prostate weight normalized by body weight. The data presented are mean ± standard error of mean. *, significant different from the Control group ($P<0.05$); **, significant from the MSC group ($P<0.05$).

All treatments appeared to be well tolerated. With the exception of two mice (one in MF5 and the other in MF50) died early in the experiment, animals in all treatment groups appeared healthy. Total body weight was recorded throughout the experiment and no significant difference was found among the groups (Fig. 1B). Mice in the M+F5

group appeared to have higher body weight, presumably due to smaller tumor burden. Consistent with clinical findings, treatment with finasteride reduced the weight of prostate in the animals (Figure 1C). This result indicates that the observed lack of effectiveness of finasteride on tumor growth inhibition is likely due to changes in tumor biology, rather than a loss of drug potency.

New Task. Combined androgen signaling blockade by emodin and finasteride in prostate cancer chemoprevention.

This new task was inspired by an observation in the Nutrition Prevention of Cancer (NPC) trial. The NPC trial showed that the protective effect of selenium was limited to patients with baseline serum selenium in the lower 2 tertiles.[2] In agreement with this observation, 78% of men in SELECT, which showed selenium supplementation did not reduce prostate cancer risk, had baseline selenium above the range that selenium provided protection in the NPC trial (<121.6 ng/ml).[3] Therefore, it is possible that individuals with high baseline selenium level will not be benefited from selenium supplementation. Therefore, we investigated the efficacy of emodin and finasteride combination in prostate cancer chemoprevention. Emodin is a phytochemical that has been shown to induce AR degradation.[4] We hypothesize that the combination of emodin and finasteride synergizes on inhibiting androgen signaling and subsequently, on inhibiting tumor cells growth.

To test this hypothesis, we performed the ARE-luciferase assay in LNCaP cells treated with emodin and finasteride. As shown in Figure 2A, the activity of ARE-luciferase was stimulated by testosterone (T). Finasteride and emodin each inhibited AR activity in a dose-dependent manner. In cells that received the combination treatment, the inhibition was significantly stronger than in cells receiving single treatments. These results was confirmed when we examined the expression of prostate specific antigen (PSA), a well-known target of AR, by real-time reverse-transcription polymerase chain reaction (qRT-PCR) (Figure 2B) and Western blotting (Figure 2C). Collectively, these results suggest a synergy between emodin and finasteride in suppressing androgen signaling in prostate cancer cells.

We next examined the efficacy of emodin and finasteride in growth arrest in LNCaP cells. Cells were treated with various concentrations of emodin and finasteride for 48 hr and cell proliferation was measured by the BrdU incorporation assay. Figure 3A shows that finasteride (1 μM) had a modest effect in inhibiting cell proliferation, whereas the inhibitory effect of emodin was dose-dependent. In all the doses tested, the combination with finasteride significant enhanced the efficacy of emodin. Apoptosis induction, which was measured by using the Cell Death Detection ELISA kit (Roche) and Western blotting for PARP, showed similar results (Figure 3, B&C).

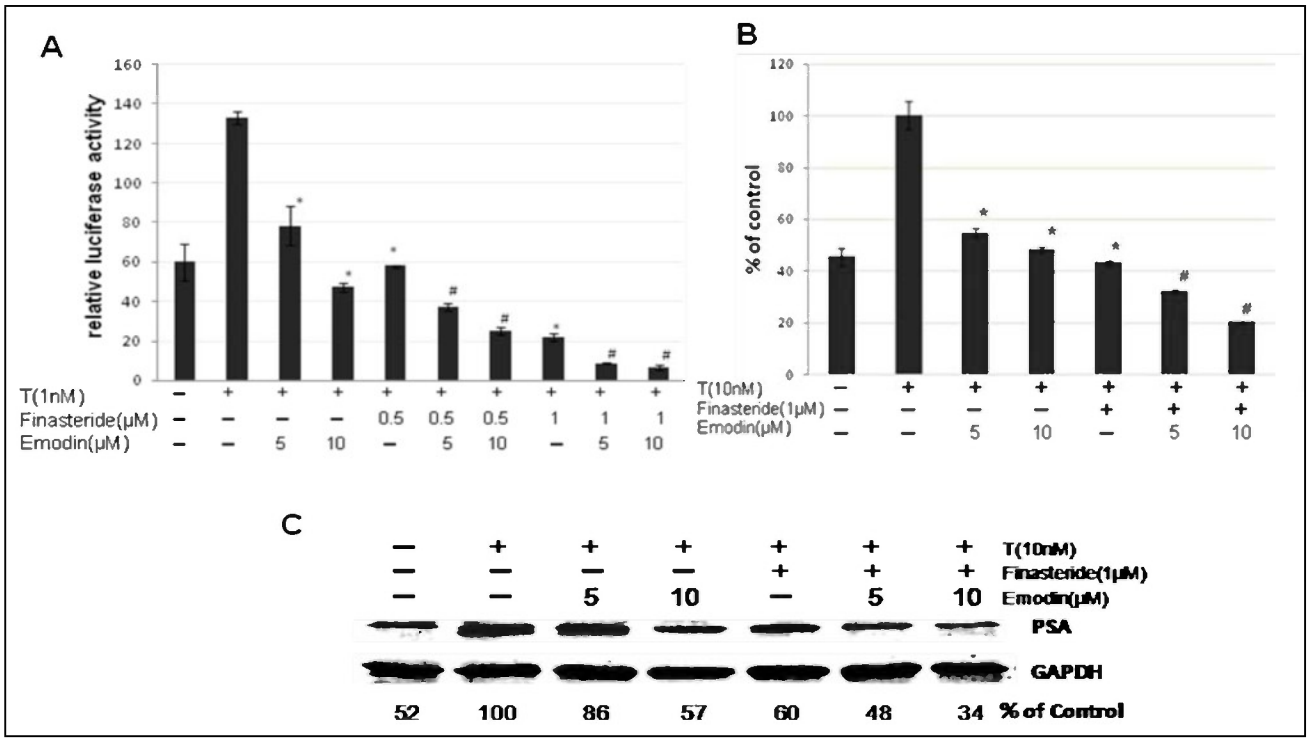

Figure 2. Suppression of androgen signaling by emodin and finasteride. A. ARE-luciferase assay. B. qRT-PCR analysis of PSA expression. C. Western blotting analysis of PSA expression. The intensity of the PSA band was normalized by that of the GAPDH. The data presented in A and B are mean ± SEM. *, statistically significant from the T-stimulated, untreated control (P<0.01); #, statistically significant from the single treatments (P<0.01).

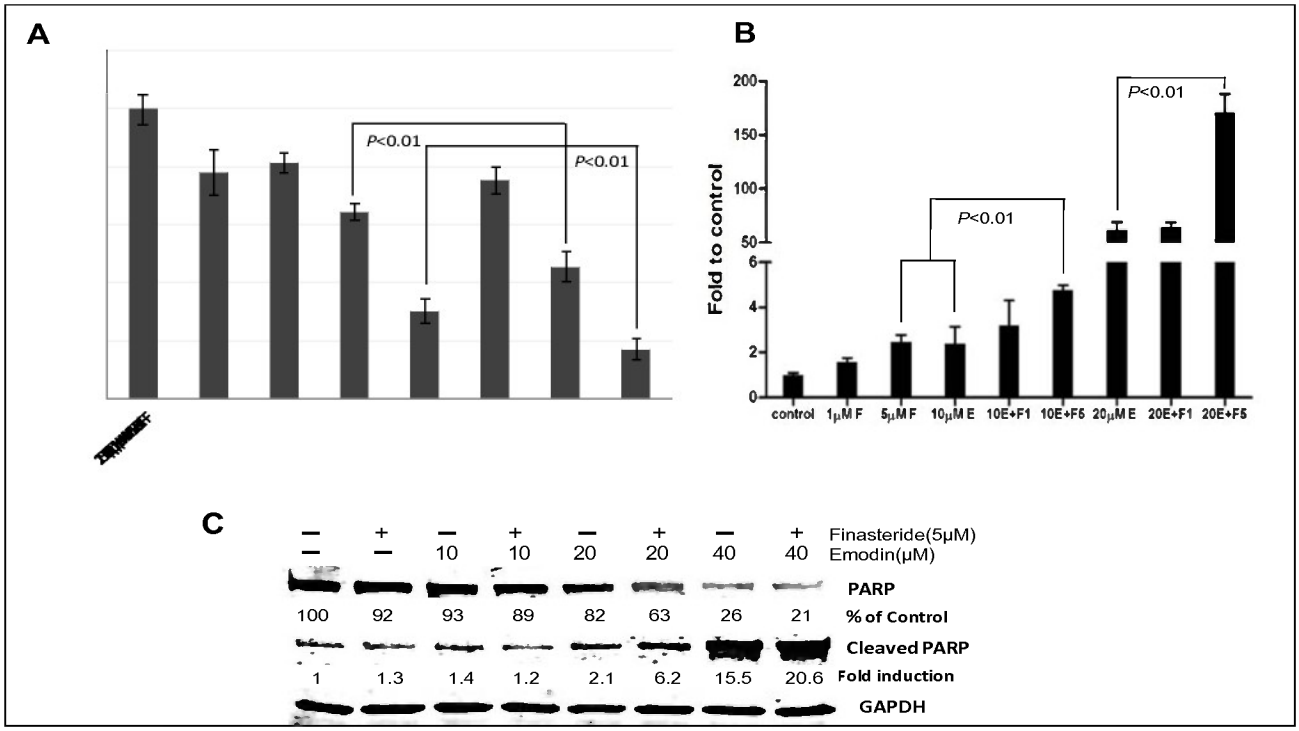

Figure 3. Effect of emodin and finasteride on growth inhibition in LNCaP cells. A. BrdU incorporation assay. B. Apoptosis assay by the Cell Death ELISA assay. C. Western blotting of PARP cleavage.

C. KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated the synergy between emodin and finasteride in suppressing androgen signaling in prostate cancer cells.

- Demonstrated the efficacy of emodin and finasteride in inhibiting cell proliferation and in inducing apoptosis in prostate cancer cells.

- The tumor xenograft study provided indications of the advantages for the combination of selenium and finasteride over single treatments, suggesting selenium may function as a modulator to increase the efficacy of finasteride and prevent drug resistance.

D. REPORTABLE OUTCOMES

Manucript

Haitao Zhang, Jian Fang, Dian Yao, Yue Wu, Clement Ip, and Yan Dong. Activation of FOXO1 Is Critical for the Anticancer Effect of Methylseleninic Acid in Prostate Cancer Cells. Submitted to *Cancer.*

Presentation

Jilin University, Changchun, Jilin, China, July 27, 2009, invited speech, "Targeting androgen signaling for prostate cancer intervention".

E. CONCLUSIONS

The results from the animal experiment showed promises in the combination of selenium and finasteride in preventing the clonal expansion of small volume, early stage prostate cancer. Finasteride, at both dose (5 and 50 mg/kg/day), was effective only for a short duration and the tumors appeared to have developed resistance to finasteride after prolonged treatment. MSC, albeit not effective when used alone, was able to enhance the efficacy of finasteride and prevent the development of resistance by the tumors. These findings have significant clinical implications with regard to the uses of selenium and finasteride as chemopreventive agents.

Due to the small sample size, the observed effects were not statistically significant. A repeat of the experiment with a larger sample size is necessary to corroborate the findings. Therefore, we request a no-cost extension for 6 months to conduct additional animal experiments.

We also demonstrated the efficacy of emodin and finasteride in suppressing androgen signaling and growth inhibition in prostate cancer cells. This finding provides another option for combined androgen blockade in prostate cancer chemoprevention.

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Activation of FOXO1 Is Critical for the Anticancer Effect of Selenium in Prostate Cancer Cells

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Running title: FOXO1 mediates the anticancer actions of selenium

Key words: selenium, chemoprevention, FOXO1, apoptosis, androgen receptor

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Abbreviation List: AR, androgen receptor; ARE, androgen response element; FOXO1, forkhead box O1; MSA, methylseleninic acid; NPC, Nutritional Prevention of Cancer; PSA, prostate specific antigen; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SELECT, selenium and vitamin E chemoprevention trial; siRNA, small interfering RNA.

ABSTRACT

Previous studies have demonstrated that physiological concentrations of selenium inhibit the growth of prostate cancer cells. The growth inhibitory effect could be attributed to cell cycle block and apoptosis induction. The current study was designed to investigate the involvement of forkhead box O1 (FOXO1) in the anticancer effect of selenium. Selenium treatment led to a rapid and robust increase of FOXO1 expression, as well as an increase of the FOXO1 transcriptional activity. Blocking FOXO1 activation by a specific small interfering RNA (siRNA) abolished apoptosis induction by selenium, suggesting FOXO1 plays a critical role in mediating the apoptotic effect of selenium. Recent studies have shown that FOXO1 and the androgen receptor (AR) antagonize the actions of each other. We examined the consequence of FOXO1 induction on AR activity. Consistent with previous reports, we found that ectopic expression of FOXO1 suppressed the transcriptional activity of AR. Furthermore, FOXO1 silencing attenuated selenium suppression of AR activity, suggesting that FOXO1 induction contributes to suppression of AR signaling by selenium. Collectively, these data suggest FOXO1 is a key mediator of the anticancer effect of selenium in prostate cancer cells.

INTRODUCTION

Prostate cancer is a significant public health problem that engenders huge medical care and human suffering costs in the United States. A number of case-control studies have demonstrated an inverse relationship between selenium status and prostate cancer risk (1-5). One of the more important studies of selenium as a chemopreventive agent is the Nutritional Prevention of Cancer (NPC) trial initiated by Larry Clark (6,7). The study was a randomized, double-blind, placebo-controlled trial involving 1312 patients (mostly men) who were recruited initially because of a history of basal cell or squamous cell carcinoma of the skin. Individuals in the treatment arm were given 200 μg selenium per day in the form of selenized yeast for a mean of 4.5 years. After a total follow-up of 8271 person-years, selenium treatment did not decrease the recurrence of these non-melanoma skin cancers. However, patients receiving the supplement showed a much lower risk of developing lung (RR=0.54), colon (RR=0.42) or prostate cancer (RR=0.37) (6,7).

Encouraged by the prostate cancer results from the NPC trial, the National Cancer Institute launched the Selenium and Vitamin E Cancer Prevention Trial (SELECT) in 2001. This is a double-blind, randomized, placebo-controlled trial to determine the efficacy of selenium, either alone or in combination with Vitamin E, in preventing prostate cancer in an average-risk cohort of over 35,000 men (8). L-selenomethionine, a major form of selenium in selenized yeast, at a daily dose of 200 μg was chosen as the formulation of selenium for this trial. An interim data analysis after a median follow-up of 5.46 years showed there was no significant change in prostate cancer risks between the three intervention groups and the placebo group, suggesting selenomethionine and vitamin E, alone or in combination, were ineffective in preventing prostate cancer in this

study population. The trial was halted in October of 2008, but the follow-up will continue for 3 more years.

The results of the SELECT have caused much controversy regarding the study design and the implication for selenium and prostate cancer prevention. Because of the study design, the SELECT could not assess the effect of selenium in men with elevated risk for prostate cancer, or in those with selenium deficiency, or in reducing the risk for advanced prostate cancer. Currently, there are two ongoing large scale clinical trials, the Prevention of Cancer by Intervention with Selenium (PRECISE) trial and the Australian Prostate Cancer Prevention Trial Using Selenium (APPOSE). The PRECISE trial, which is conducted in 3 countries in Europe, uses selenized yeast at doses of 100, 200, or 300 μg/day (9). The APPOSE study will test the impact of 200 μg of daily selenium supplementation on prostate cancer incidence in a high risk population (10). It is worth pointing out that both studies are done in countries that selenium content in food is lower than that in the US (11). Therefore, these studies will complement the SELECT and paint a more complete picture on the role of selenium in prostate cancer intervention.

The formulation and dose of selenium used in the SELECT study have also been hot topics of debate. Monomethylated forms of selenium, including methylseleninic acid (MSA) and methylselenocysteine (MSC), are second generation selenium compounds. Metabolically, these compounds are very different from selenomethionine, the formulation used in the SELECT. MSA and MSC can be easily converted to methylselenol, which is considered to be the critical metabolite for the anticancer activity of selenium (12,13). Methylselenol is highly reactive and difficult to prepare. Therefore, the proximal precursors including MSA and MSC are used to provide a steady stream of methylselenol. Selenomethionine, on the other hand, can be incorporated

nonspecifically into proteins in place of methionine (12). Due to its compartmentation into tissue proteins, selenomethionine is not as readily available as MSA and MSC for further metabolism. The metabolism of selenomethionine to methylselenol requires at least 5 transsulfuration steps and the action of thiol methyltransferase (12,14,15). Studies published prior to and after the start of the SELECT study have showed that MSA and MSC have stronger anticancer activities than selenomethionine (16-18). The conversion of MSC to methylselenol requires the action of β-lyase, whereas MSA can be easily reduced to methylselenol through non-enzymatic reactions involving glutathione (GSH) or NADPH (19). Due to the fact that epithelial cells express low level of β-lyase, MSA is 10 times more potent than MSC in affecting biological processes *in vitro* (13). MSA is widely accepted to be the best reagent for delineating the molecular action of selenium in cell culture studies (20-23). It also has excellent anticancer activity in animals (13,24,25). We conducted all the experiments in the current study using MSA.

In view of the above information, we believe that selenium is still a promising agent for prostate cancer chemoprevention when used in discretion. Unraveling its mechanism of action is urgent and will no doubt be helpful in selecting the appropriate population of individuals for a more rational design of selenium intervention trial. We and others have previously profiled selenium-induced gene expression changes in prostate cancer cells (26,27). Based on the datasets generated from the microarray studies, we conducted a systematic data mining analysis, taking advantage of several publicly available clinical prostate cancer datasets, in order to gain new insights into novel molecular targets that may be relevant to the anticancer activity of selenium (28). The analysis drew our attention to FOXO1. We found that the expression of FOXO1 is consistently decreased in a large number of prostate cancer specimens, and the

microarray analyses showed selenium up-regulates the expression of FOXO1 (28). FOXO1 is a member of the FOXO family of transcription factors that induces the expression of pro-apoptotic genes including Fas ligand (29,30), bcl-2 family proteins (31-33), and TRAIL (34). FOXO1 is also involved in cell cycle regulation (35). FOXO1 is phosphorylated and suppressed by AKT (36,37), which is an important survival molecule for prostate cancer. In prostate cancer cells, AR interacts with FOXO1 and inhibits its activation of downstream targets (38). The current study was designed to examine the role of FOXO1 in mediating the anticancer effect of selenium.

RESULTS

MSA induces FOXO1 expression

We first performed qRT-PCR and Western blotting to confirm the modulation of FOXO1 by MSA in LNCaP cells, as first noted from our microarray analysis (28). Cells were treated with 10 μM MSA for various lengths of time before they were lysed for RNA and protein purification. The qRT-PCR results are shown in Fig. 1A. Induction of FOXO1 mRNA was observed as early as 1 hr after exposure to MSA, suggesting that FOXO1 is a proximal target of MSA. The mRNA level peaked at 2 hr, then declined gradually with time, but still remained elevated at 24 hr. Western blotting of FOXO1 was carried out in LNCaP and LAPC-4 cells (Fig. 1B). No change in protein level was detected until at least after 3 hr. Thus the increases of FOXO1 protein appeared to lag behind the increases of the message, although the protein signal was decidedly stronger by 6 hr in cells treated with MSA.

MSA induces the transcriptional activity of FOXO

As mentioned in Introduction, FOXO1 is a transcription factor. In order to study the effect of MSA on the activity of FOXO1 as a transcription factor, we transiently transfected LNCaP and LAPC-4 cells with a luciferase reporter construct, p3xIRS-luc. This construct has 3 tandem repeats of a FOXO1 binding element, the insulin-responsive sequence (IRS), inserted upstream of the minimal thymidine kinase promoter (37). It is widely used as an indicator of the transcriptional activity of FOXO proteins. As shown in Fig. 2A, the transcriptional activity of this reporter construct was induced by approximately 2-fold in LNCaP cells after 6 hr of treatment with 10 μM MSA. A pronounced induction (> 5-fold) was observed in LNCaP after 16 hr of treatment (P<0.01). Nearly identical results were obtained in LAPC-4 cells (Fig 2B).

FOXO1 gene silencing blocks MSA-induced apoptosis

MSA has been shown to induce apoptosis in prostate cancer cells by several groups, including ours (26,39-41). The experiments described above suggested that MSA induces the FOXO1 signaling pathway, which is known to positively regulate apoptosis. To establish the role of FOXO1 in MSA-induced apoptosis, we employed the RNA interference technique to knockdown the expression of FOXO1. A commercially available siRNA targeting FOXO1 was obtained. To confirm the specificity of the siRNA, we performed a Local Alignment Search Tool (BLAST) against the entire human transcriptome using the sequence provided by the manufacturer. With the exception of FOXO1, the search identified no other homology with the siRNA sequence, including other FOXO members. When introduced into LNCaP cells, the FOXO1 siRNA, named siFOXO1 hereafter, was able to decrease the baseline expression of FOXO1 by approximately 50% (Fig 3A). Consistent with our previous finding, a 2-fold induction of FOXO1 was observed when the cells were treated with 10 μM MSA for 24 hr (comparing

columns 1 and 3). siFOXO1 was able to abolish this induction by MSA (comparing columns 3 and 4).

Apoptosis was quantitated in siRNA-transfected and MSA-treated cells by using an ELISA-based method. The result is shown in Fig. 3B. In general, the level of apoptosis in these cells correlated well with the expression level of FOXO1 (Fig. 3A), confirming that FOXO1 plays an important role in apoptosis regulation. More importantly, when the induction of FOXO1 was blocked by the addition of siFOXO1, no induction of apoptosis was observed (Fig 4B, comparing columns 1 and 4). These results suggest that FOXO1 is a key mediator of apoptosis induction by MSA.

FOXO1 activation suppresses AR *trans*-activation

To examine the effect of FOXO1 activation on the transcriptional activity of AR, we transiently co-transfected LNCaP cells with a reporter construct containing 3 repeats of the androgen response element (ARE) ligated in tandem to the luciferase reporter, together with a FOXO1 expression vector, pcDNA3-FKHR, or the empty vector. The ARE-luciferase reporter assay is commonly used to assess the *trans*-activating activity of AR. Following transfection, cells were exposed to 1 nM R1881, a synthetic androgen, for 6 or 16 hr before they were lysed for luciferase assay. As shown in Fig. 4, the AR transcriptional activity was greatly stimulated by the addition of the ligand. In the presence of ectopically expressed FOXO1, the induction was significantly diminished (Fig 4, comparing columns 2 and 4 for both time points). Therefore, our results confirmed published studies showing that FOXO1 activation suppresses AR signaling (42-45).

FOXO1 induction contributes to AR suppression by MSA

It has been found previously that selenium is a potent suppressor of AR signaling (10,27,46,47). The mechanisms involved in suppression of AR signaling by selenium include reduction in AR mRNA transcription (46,47) and stability, increase in AR protein turnover, reduction in AR translocation, inhibition of coactivator recruitment, and increased corepressor recruitment to the promoters of AR-regulated genes (48). The result from the previous section prompted us to investigate whether FOXO1 induction is a contributing factor for AR suppression by selenium. Once again, we employed the gene knockdown approach. LNCaP cells were co-transfected with the ARE-luciferase construct and siFOXO1, and treated with 0 or 10 μM MSA. In the presence of the scrambled oligo, MSA suppressed AR activity by approximately 70% (Fig. 5). This is in line with our previous observations (49). However, when FOXO1 was silenced, the suppression was attenuated to about 60% ($P<0.01$). This was further confirmed when we examined the modulation of PSA expression by MSA in the presence or absence of siFOXO1 (data not shown). These results are in agreement with previous studies showing that selenium suppresses AR signaling through a multitude of mechanisms and identified FOXO1 activation as a novel mechanism contributing to the inhibition of AR *trans*-activation by selenium.

DISCUSSION

Despite the protective effect of selenium against prostate cancer demonstrated by the NPC study and several studies which showed selenium is very effective in switching off androgen signaling, recent results from the Selenium and Vitamin E Cancer Prevention Trial (SELECT) showed that selenium, alone or in combination with vitamin E, did not prevent prostate cancer in a randomized trial of 33,000 men at average risk (50) .

Several potential reasons have been discussed to explain the discrepancy of the findings in SELECT and the NPC trial. In addition to the dose and formulation of selenium used in the trial, one important consideration is the baseline selenium level. The NPC trial showed that the protective effect of selenium was limited to patients with baseline serum selenium in the lower 2 tertiles (7). The average baseline selenium level of the participants in SELECT was much higher than that observed in the NPC study. In fact, 78% of men in SELECT had baseline selenium above the range that selenium provided protection in the NPC trial (<121.6 ng/ml) (50). Another important consideration is how selenium exerts its anticancer activity. The Physicians' Health Study demonstrated an inverse association of plasma selenium level with risk of advanced prostate cancer, not localized prostate cancer, suggesting selenium might function by slowing down tumor progression (4). In view of the above information, we believe that the negative finding by SELECT should not be simply interpreted as selenium is ineffective against prostate cancer. Instead, the outcome of this trial, as well as those of several recently published clinical trials (51-53), may indicate that it is difficult to find a single chemoprevention strategy which can benefit the general population. There is an urgent need to re-evaluate all the pre-clinical and clinical evidence to identify the subset of patients that are most likely to benefit from selenium supplementation.

This report is the first to show that selenium induces the expression of FOXO1. The elevated expression is accompanied by an increase of the FOXO transcriptional activity. We further demonstrated that FOXO1 is a key mediator of apoptosis induction by selenium. The above conclusion is supported by the following observations. First, FOXO1 induction occurred very early following selenium treatment, suggesting that FOXO1 is a proximal target of selenium. Second, selenium failed to induce apoptosis when FOXO1 stimulation was abolished by the addition of a FOXO1-specific siRNA.

There are two major cell death signaling pathways, one triggered through death receptors (the extrinsic pathway), and the other through the mitochondria (the intrinsic pathway). A signature of the intrinsic pathway is the release of cytochrome C from the mitochondria, which is regulated by the Bcl-2 family of proteins. As a pro-apoptotic member of the Bcl-2 family, Bim functions by antagonizing the actions of the anti-apoptotic Bcl-2 and Bcl-XL. Both TRAIL and TRADD are associated with the extrinsic pathway. Selenium has been shown to activate caspases that are involved in both the intrinsic and extrinsic apoptosis signaling pathways (39,41). We are currently working on identifying the pro-apoptotic targets of FOXO1 that are induced by selenium. In addition to its role in regulating apoptosis, FOXO1 also plays an important role in cell cycle control. It up-regulates the expression of p27 (35,54) and down-regulates the expression of cyclins D1 and D2 (55,56), a pattern consistent with the G1 cell cycle block by selenium (26,57). Therefore, it is possible that FOXO1 also mediates the cell cycle effects of selenium. Research along this line is currently ongoing in our laboratories.

Several mechanisms could account for the induction of FOXO1 signaling by selenium. One is through the induction of FOXO1 expression, as evidenced by the increased transcript and protein levels following selenium treatment. It has been shown that AR interacts and suppresses the activity of FOXO1 in prostate cancer cells (38,58). Another potential mechanism of selenium activation of FOXO1 is through decreasing AR expression and thereby relieving the inhibition of FOXO1 by AR. This is supported by the fact that ectopic expression of AR could attenuate the induction of FOXO1 activity by selenium (data not shown). Yet, there might be a third mechanism by which selenium induces FOXO1. A key regulator of cellular FOXO1 activity is Akt, an important survival molecule for many cancer types, including prostate cancer. Akt phosphorylates FOXO1,

which leads to nuclear exclusion and proteosomal degradation of FOXO1 (37). Selenium has been shown to suppress the PI3K-Akt signaling pathway (40,59-61). Therefore, it is possible that relieving the suppression by Akt may contribute to selenium induction of FOXO1. Further experimental evidence is needed to support this hypothesis.

In agreement with previous reports (42-45), our data showed increased abundance of FOXO1 leads to decreased AR activity. Together with the well-documented AR inhibition of FOXO1 activity, it appears that in prostate cancer cells, the AR and FOXO1 signaling pathways antagonize the action of each other. The outcome is likely determined by the relative abundance of AR and FOXO1 proteins. When AR signaling dominates, the growth inhibitory signals conveyed by FOXO1 are muted, and the cells undergo proliferation. On the other hand, when FOXO1 signaling dominates, the antiproliferative and proapoptotic signalings prevail. When prostate cancer cells are exposed to selenium, AR signaling is suppressed whilst FOXO1 signaling is stimulated. By doing so, selenium could shift the balance heavily in favor of FOXO1, leading to cell cycle arrest and apoptosis. Therefore, modulating the crosstalk between AR and FOXO1 could be the key mechanism underlining the anticancer effect of selenium in the prostate.

In summary, the work described herein demonstrates that selenium activates FOXO1 signaling pathway. FOXO1 plays a critical role in mediating the apoptotic activity of selenium, and also contributes to the suppression of androgen signaling by selenium. This study enhances our understanding of the molecular mechanisms of the anticancer activity of selenium, which will be critical for designing future prostate cancer intervention studies with selenium.

MATERIALS AND METHODS

Materials

MSA was purchased from PharmaSe (Lubbock, TX). Fetal bovine serum, RPMI 1640, and the Lipofectamine PLUS transfection reagents were purchased from Invitrogen (Carlsbad, CA). Immobilon PVDF membrane was purchased from Millipore (Bedford, MA) and ECL Western blotting detection reagent from Amersham Pharmacia Biotech (Arlington Heights, IL). For Western blotting analysis, the anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Chemicon (Temecula, CA) and anti–FOXO1 was from Cell Signaling (Danvers, MA). The Cell Death Detection ELISA kit was purchased from Roche Applied Science (Indianapolis, IN). The p3XIRS-luc reporter construct was kindly provided by Dr. Kun-Liang Guan at the University of Michigan, and the pcDNA3-FKHR expression vector was obtained from Dr. Frederic G. Barr at the University of Pennsylvania. The pcDNA3-AR-FL expression vector was a gift from Dr. Shuyun Yeh at the University of Rochester.

Cell Culture and Treatment

The human LNCaP prostate cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). The LAPC-4 cell line was provided by Dr. Charles L. Sawyers at the University of California at Los Angeles Jonsson Comprehensive Cancer Center. Both LNCaP and LAPC-4 express AR and require androgen for their growth. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 unit/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM of glutamine. In some experiments, cells were cultured in an androgen-defined

condition by using charcoal-stripped FBS in the presence of 1 nM R1881 (a potent synthetic androgen). Treatment with MSA usually began at 72 hr after seeding, when the cultures were 60-80% confluent.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The PCR primers and Taqman probes for β-actin, FOXO1, and AR were Assays on-Demand products from Applied Biosystems (Foster City, CA). The PCR conditions were as follows: an initial incubation at 50°C for 2 minutes, then a denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantitation of gene expression was done using the comparative CT ($\Delta\Delta C_T$) method (62). Details of the procedure were described in our previous publication (49).

Transient Transfection and Reporter Gene Assay

Supercoiled plasmid DNAs were prepared by the Qiagen column procedure (Qiagen, Valencia, CA). Twenty-four hours before transfection, cells were trypsinized and seeded at a density to reach 90-95% confluency at the time of transfection. Transient transfection was carried out by using the Lipofectamine™ and Plus™ reagents (Invitrogen, Carlsbad, CA) per instruction of the manufacturer. After incubating with the transfection mixture for 3 hr, the cells were trypsinized and re-plated in triplicate into 6-well plates to achieve equal transfection efficiency. The cells were allowed to attach overnight before 10 μM MSA was added to the culture medium. At 6 or 16 hr following treatment, cells were lysed with 1X Passive Lysis Buffer (Promega, Madison WI), and the luciferase activity was assayed by using the Luciferase Assay System (Promega). Protein concentration in the cell extract was determined by using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Luciferase activities were normalized to the

protein concentration of the same sample. The transfection experiments were repeated three times.

Gene silencing with siRNA

A siRNA designed to target FOXO1 (Cat. # HSS103719) and a matching negative control oligonucleotide were purchased from Invitrogen. These oligonucleotides were transiently transfected into LNCaP cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 hr post transfection, 10 μM MSA was added to the culture medium and the cells were treated for an additional 24 hr. RNA was prepared from the cells and qRT-PCR was performed to determine the efficiency of gene silencing.

Statistical Analysis

The Student's *t* test was used to determine significant differences between different groups. Unless otherwise indicated, $P<0.05$ was considered statistically significant. All analyses were two-tailed.

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FIGURE LEGENDS

Figure 1. Effect of MSA on FOXO1 expression. A. Change of FOXO1 mRNA in LNCaP cells as a function of time of MSA treatment, determined by qRT-PCR. The results are shown as mean ± SEM. B. Western analysis of FOXO1 protein level as a function of time of MSA treatment, in both LNCaP and LAPC-4 cells. The band intensity was quantified by volume densitometry and normalized to that of GAPDH. The results were expressed as fold induction over untreated.

Figure 2. ***Induction of FOXO transcriptional activity by MSA.*** LNCaP (A) and LAPC-4 (B) cells were transfected with the p3XIRS-luc construct and treated with 10 μM MSA for the indicated times. At the end of treatment, cells were lysed for luciferase assay. Total protein concentration was also determined and used to normalize the luciferase reading. The results were expressed as mean ± SEM. *, $P<0.05$.

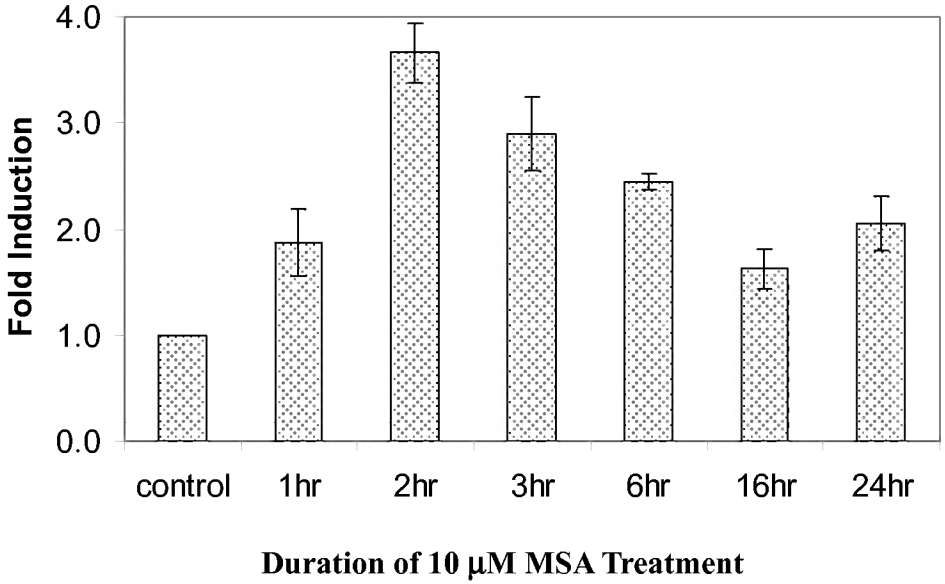
Figure 3. ***Effect of FOXO1 gene silencing on MSA-induced apoptosis.*** A, qRT-PCR analysis of FOXO1 expression in cells transfected with siRNAs and treated with or without MSA. The data were expressed as fold relative to the scramble, untreated control. B. Quantitation of apoptotic cell death by an ELISA method.

Figure 4. ***Increased expression of FOXO1 reduced the transcriptional activity of AR.*** LNCaP cells were co-transfected with the ARE-luciferase reporter construct and either the pcDNA3-FKHR or the pcDNA3 vector, and treated with 1 nM R1881 for the indicated times.

Figure 5. ***FOXO1 knockdown attenuated the suppression of AR trans-activation by MSA.*** LNCaP cells were co-transfected with the ARE-luciferase construct and either the scrambled control or siFOXO1, and treated with 10 μM MSA for 24 hr. The luciferase reading was normalized by protein concentration. The experiment was done 3 times and the results were expressed as mean percent inhibition ± SEM.

Figure 1

A. qRT-PCR



B. Western

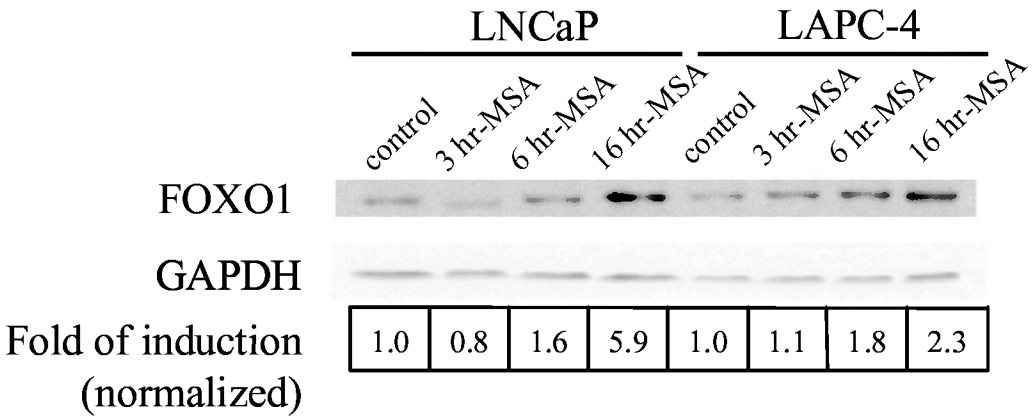
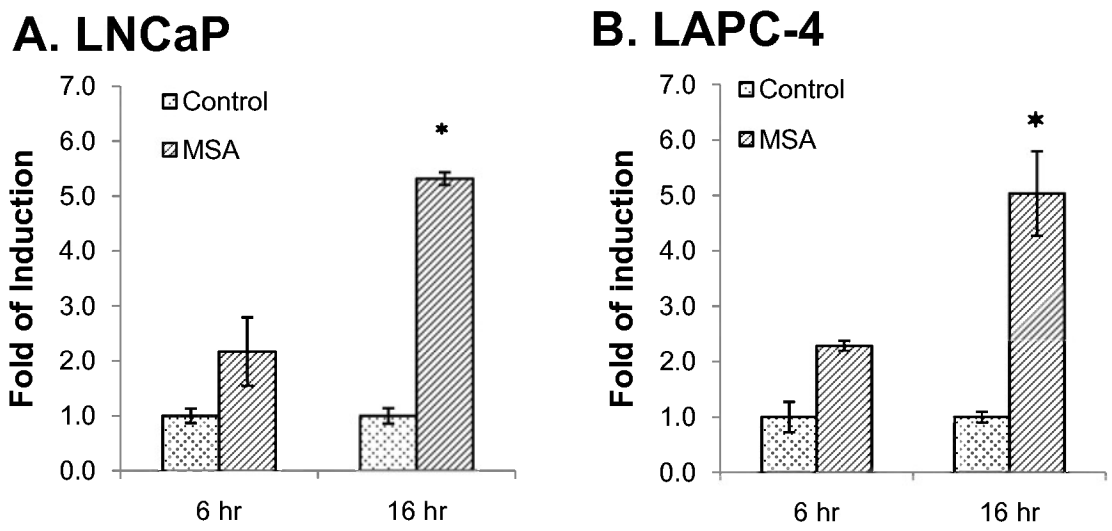
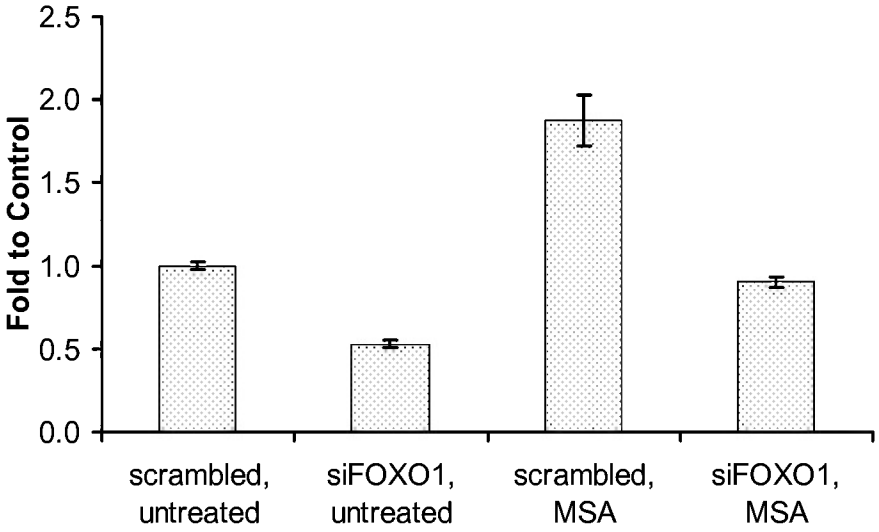


Figure 2


A. LNCaP
Fold of Induction
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Control
MSA
*
6 hr
16 hr
B. LAPC-4
Fold of induction
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Control
MSA
*
6 hr
16 hr

A. qRT-PCR on FOXO1 expression



B. Cell Death ELISA

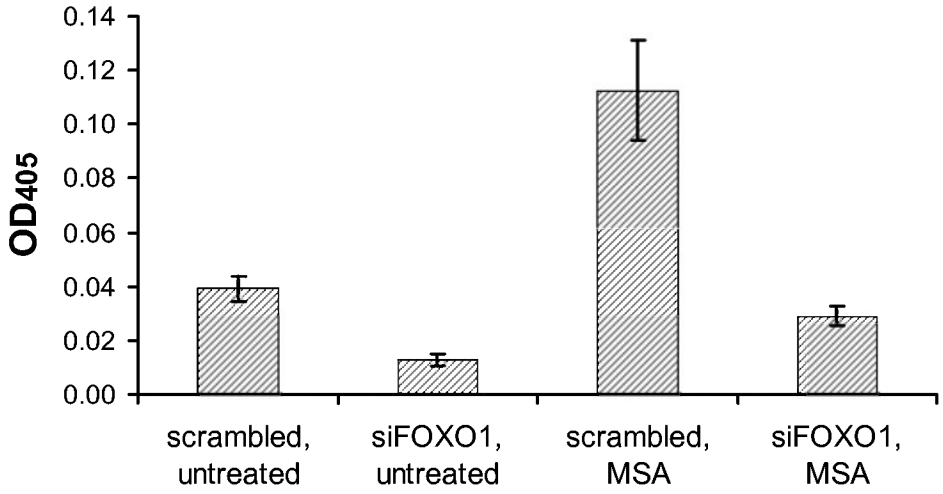
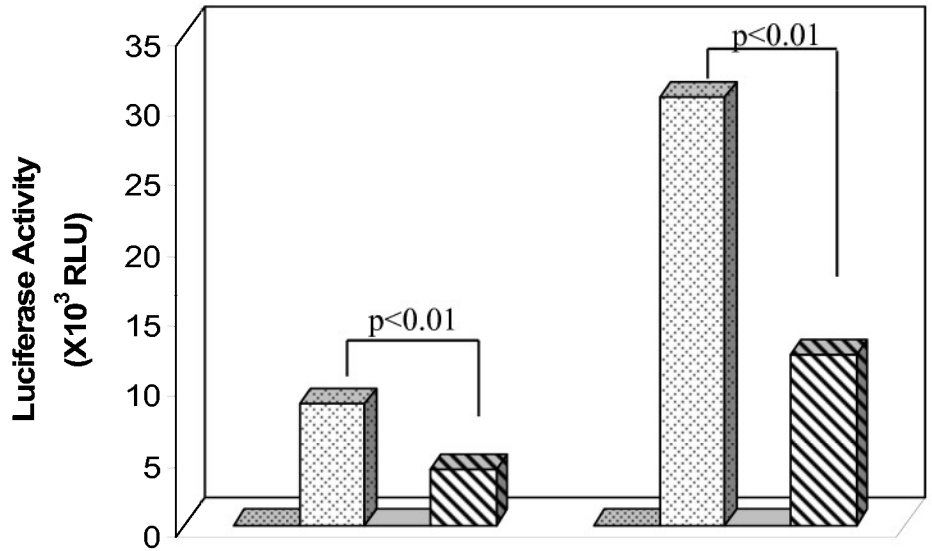


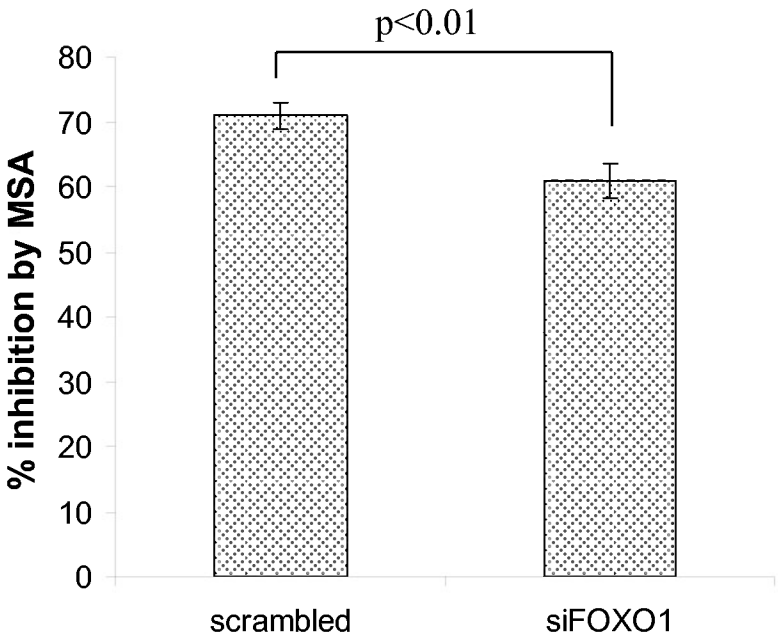
Figure 3

Figure 4



R1881	-	+		-	+		-	+		-	+
Time (hr)	6	6	6	6			16	16	16	16
FOXO1	-	-		+	+		-	-		+	+
pCDNA3.1	+	+		-	-		+	+		-	-

Figure 5


p<0.01
% inhibition by MSA
80
70
60
50
40
30
20
10
0
scrambled
siFOXO1